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Purification and characterization of a newly identified growth factor specific for epithelial cells

(mitogen/keratinocyte/protein purification/heparin binding/N-terminal sequence)

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ABSTRACT **A growth factor specific for epithelial cells was identified in conditioned medium of a human embryonic lung fibroblast cell line. The factor, provisionally termed keratinocyte growth factor (KGF) because of its predominant activity on this cell type, was purified to homogeneity by a combination of ultrafiltration, heparin-Sepharose affinity chromatography, and hydrophobic chromatography on a C_4 reversed-phase HPLC column. KGF was both acid and heat labile and consisted of a single polypeptide chain of ≈28 kDa. Purified KGF was a potent mitogen for epithelial cells, capable of stimulating DNA synthesis in quiescent BALB/MK epidermal keratinocytes by >500-fold with activity detectable at 0.1 nM and maximal at 1.0 nM. Lack of mitogenic activity on either fibroblasts or endothelial cells indicated that KGF possessed a target cell specificity distinct from any previously characterized growth factor. Microsequencing revealed an amino-terminal sequence containing no significant homology to any known protein. The release of this growth factor by human embryonic fibroblasts raises the possibility that KGF may play a role in mesenchymal stimulation of normal epithelial cell proliferation.**

Growth factors are important mediators of intercellular communication. These potent molecules are generally released by one cell type and act to influence proliferation of other cell types (1). Interest in growth factors has been heightened by evidence of their potential involvement in neoplasia. The v-*sis* transforming gene of simian sarcoma virus encodes a protein that is homologous to the B chain of platelet-derived growth factor (2, 3). Moreover, a number of oncogenes are homologues of genes encoding growth factor receptors (4). Thus, increased understanding of growth factors and their receptor-mediated signal-transduction pathways is likely to provide insights into mechanisms of both normal and malignant cell growth.

Recognizing that the vast majority of human malignancies are derived from epithelial tissues (5), we sought to identify growth factors specific for these cell types. In this communication, we report the purification to homogeneity of such a growth factor released by a human embryonic lung fibroblast line. Our demonstration of its unique N-terminal amino acid sequence and epithelial cell specificity distinguishes this mitogen from any previously described growth factor.

METHODS AND MATERIALS

Cell Culture. M426 human embryonic fibroblasts (6), BALB/MK mouse epidermal keratinocytes (7), and NIH 3T3 mouse embryonic fibroblasts (8) were established in this laboratory. CCL208 rhesus monkey bronchial epithelial cells (9) were obtained from the American Type Culture Collection, and the B5/589 human mammary epithelial cell line, prepared as described (10), was a gift from M. Stampfer (Lawrence Berkeley Laboratory). Primary cultures of human saphenous vein endothelial cells were prepared and maintained as described elsewhere (11). Epidermal growth factor (EGF) and insulin were from Collaborative Research, and transforming growth factor type α (TGF-α) was from Genentech. Acidic fibroblast growth factor (aFGF) and basic FGF (bFGF) were gifts from J. Abraham (California Biotechnology, Inc.). Media and serum were from GIBCO, Biofluids (Rockville, MD), or the National Institutes of Health media kitchen.

Preparation of Conditioned Medium. An early passage of M426 fibroblasts was plated onto 175-cm^2 T flasks and grown to confluence over 10–14 days in Dulbecco's modified Eagle's medium (DMEM; GIBCO) supplemented with 10% (vol/vol) calf serum (GIBCO). Once confluent, the monolayers were cycled weekly from serum-containing to serum-free medium, the latter consisting of DMEM alone. The cells were washed twice with 5 ml of phosphate-buffered saline prior to addition of 20 ml of DMEM. After 72 hr, culture fluids were collected and replaced with 35 ml of serum-containing medium. The conditioned medium was stored at −70°C until further use.

Ultrafiltration. Approximately 10 liters of conditioned medium was thawed, prefiltered through a 0.50-μm filter (Millipore HAWP 142 50), and concentrated to 200 ml by using the Pellicon cassette system (Millipore XX42 00K 60) and a cassette having a 10-kDa molecular mass cutoff (Millipore PTGC 000 05). After concentration, the sample was subjected to two successive rounds of dilution with 1 liter of 20 mM Tris·HCl, pH 7.5/0.3 M NaCl, each followed by ultrafiltration with the Pellicon system. Activity recovered in the retentate was either immediately applied to the heparin-Sepharose resin or stored at −70°C.

Heparin-Sepharose Affinity Chromatography (HSAC). The retentate from ultrafiltration was loaded onto heparin-Sepharose resin (Pharmacia) that had been equilibrated in 20 mM Tris·HCl, pH 7.5/0.3 M NaCl. The resin was washed extensively until the absorbance had returned to baseline and then was subjected to a linear-step gradient of increasing NaCl concentration. After aliquots were removed from the fractions for the thymidine incorporation bioassay, selected fractions were concentrated 10- to 20-fold with a Centricon-10 microconcentrator (Amicon) and stored at −70°C.

Abbreviations: KGF, keratinocyte growth factor; EGF, epidermal growth factor; TGF-α, transforming growth factor α; aFGF, acidic fibroblast growth factor; bFGF, basic fibroblast growth factor; HSAC, heparin-Sepharose affinity chromatography; RP-HPLC, reversed-phase HPLC.

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Reversed-Phase HPLC (RP-HPLC). Active fractions (0.6 M NaCl pool) from the HSAC were thawed, pooled, and further concentrated with the Centricon-10 microconcentrator to a final volume of ≤200 μl. The sample was loaded onto a Vydac C_4 HPLC column (The Separations Group) that had been equilibrated in 0.1% trifluoroacetic acid (Fluka)/20% acetonitrile (Baker, HPLC grade) and eluted with a linear gradient of increasing acetonitrile. Aliquots for the bioassay were immediately diluted in a 10-fold excess of 20 mM Tris·HCl (pH 7.5) containing bovine serum albumin (fraction V, Sigma) at 50 μg/ml. The remainder of the sample was dried in a Speed-Vac (Savant) in preparation for structural analysis.

Molecular-Sieve HPLC. Approximately 50 microliters of the twice concentrated heparin-Sepharose fractions were loaded onto a TSK G3000SW Glas-Pac column (LKB) that had been equilibrated in 20 mM Tris·HCl, pH 6.8/0.5 M NaCl. The sample was eluted in this buffer at a flow rate of 0.4 ml/min. After removing aliquots for the bioassay, the fractions were stored at −70°C.

$NaDodSO_4$/PAGE. Polyacrylamide gels were prepared with $NaDodSO_4$ by the procedure of Laemmli (12). Samples were boiled for 3 min in the presence of 2.5% (vol/vol) 2-mercaptoethanol. The gels were fixed and silver-stained (13) by using the reagents and protocol from Bio-Rad. Molecular weight markers were from Pharmacia.

Mitogenic Assay. DNA synthesis was measured as described elsewhere (14) with a few modifications. Ninety-six-well microtiter plates (Falcon no. 3596) were precoated with human fibronectin (Collaborative Research) at 1 μg/cm² prior to seeding with indicator cells. Incorporation of [³H]-thymidine was monitored during a 6-hr period beginning 16 hr after addition of samples.

Proliferation Assay. Culture dishes (35 mm) were precoated sequentially with poly(D-lysine) (20 μg/cm²; Sigma) and human fibronectin and then were seeded with ≈2.5 × 10⁴ BALB/MK cells. The basic medium was 1:1 (vol/vol) Eagle's low-Ca^{2+} minimal essential medium/Ham's F-12 medium supplemented with 5 μg of transferrin per ml, 30 nM Na_2SeO_3, and 0.2 mM ethanolamine (Sigma). Medium was changed every 2 or 3 days. After 10 days, the cells were fixed in formalin (Fisher) and stained with Giemsa (Fisher).

Protein Microsequencing. Approximately 4 μg (≈150 pmol) of protein from the active fractions of the C_4 column were redissolved in 50% trifluoroacetic acid and loaded onto an Applied Biosystems gas-phase protein sequenator. Twenty rounds of Edman degradation were carried out, and identifications of amino acid derivatives were made with an automated on-line HPLC column (model 120A, Applied Biosystems).

RESULTS

Growth Factor Detection and Isolation. Preliminary screening of conditioned media from various cell lines indicated that media from some fibroblast lines contained mitogenic activities detectable on both BALB/MK epidermal keratinocytes and NIH 3T3 embryonic fibroblasts. Whereas boiling destroyed the activity on BALB/MK cells, mitogenic activity on NIH 3T3 cells remained intact. Based on the known heat stability of EGF (15) and TGF-α (16), we reasoned that BALB/MK mitogenic activity might be due to a different agent. M426, a human embryonic lung fibroblast line, was selected as the best source of this activity for purification of the putative growth factor(s).

Ultrafiltration with the Pellicon system provided a convenient way of reducing the sample volume to a suitable level for subsequent chromatography. HSAC, which has been used in the purification of other growth factors (17–22), provided the most efficient purification step. While estimates of specific recovered activity were uncertain at this stage because of the likely presence of other factors, the apparent yield of activity was 50–70% with a corresponding enrichment of ≈1000-fold. More than 90% of the BALB/MK mitogenic activity was eluted with 0.6 M NaCl (Fig. 1) and was not associated with any activity on NIH 3T3 cells (data not shown). Because of the reproducibility of the HSAC pattern, active fractions could be identified presumptively on the basis of the gradient and absorption profile. Prompt concentration of 10- to 20-fold with the Centricon-10 microconcentrator was found to be essential for stability, which could be maintained subsequently at −70°C for several months.

Final purification was achieved by RP-HPLC with a C_4 Vydac column, a preparative method suitable for amino acid sequence analysis. While the yield of activity from the C_4 step was usually only a few percent, this loss could be attributed to the solvents used. In other experiments, exposure to 0.1% trifluoroacetic acid/50% (vol/vol) acetonitrile for 1 hr at room temperature reduced the mitogenic activity of the preparation by 98%. Nonetheless, a single peak of BALB/MK stimulatory activity was obtained (Fig. 2), coinciding with a distinct peak in the absorption profile. The peak fractions contained a single band on the silver-stained gel (Fig. 2*B*), and the relative mitogenic activity (Fig. 2*C*) correlated well with the intensity of the band across the activity profile.

An alternative step, using molecular-sieve chromatography with a TSK G3000SW GlasPac column run in aqueous solution near physiologic pH, resulted in a major peak of activity in the BALB/MK bioassay (Fig. 3). This preparation was almost as pure as the one obtained from RP-HPLC as

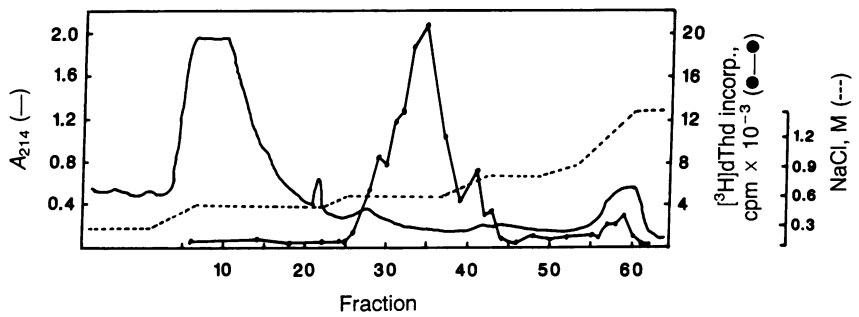

FIG. 1. HSAC of conditioned medium from M426 human embryonic fibroblasts. Approximately 150 ml of ultrafiltration retentate derived from 5 liters of M426-conditioned medium was loaded onto a heparin-Sepharose column (6-ml bed volume) in 1 hr. After the column was washed with 150 ml of the equilibration buffer (20 mM Tris·HCl, pH 7.5/0.3 M NaCl), the retained protein (<5% total protein in retentate) was eluted with a modified linear gradient of increasing NaCl concentration. Fraction size was 3.8 ml, and flow rate during gradient elution was 108 ml/hr. Two microliters of the indicated fractions was transferred to microtiter wells and diluted to a final volume of 0.2 ml for assay of [³H]thymidine incorporation (incorp.) in BALB/MK cells.

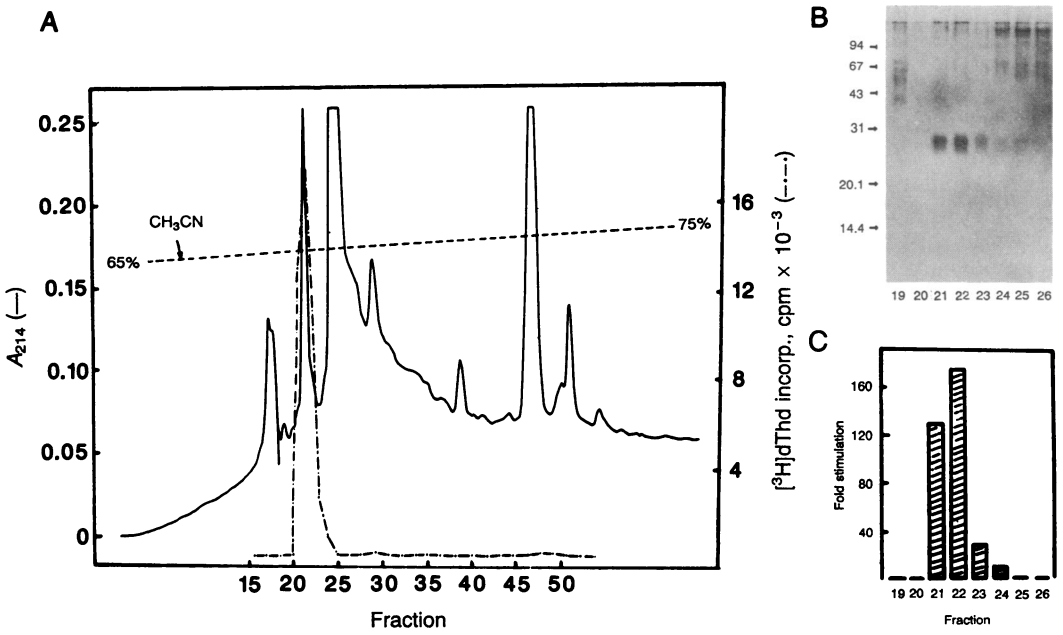

FIG. 2. (*A*) C_4 RP-HPLC of BALB/MK mitogenic activity. Active fractions eluted from the heparin-Sepharose column with 0.6 M NaCl were processed with the Centricon-10 microconcentrator and loaded directly onto a C_4 Vydac column (4.6 × 250 mm) that had been equilibrated in 0.1% trifluoroacetic acid/20% acetonitrile. After the column was washed with 4 ml of equilibration buffer, the sample was eluted with a modified linear gradient of increasing percentage of acetonitrile. Fraction size was 0.2 ml, and flow rate was 0.5 ml/min. Aliquots for the assay of [3H]thymidine incorporation (incorp.) in BALB/MK cells were promptly diluted 1:10 with 50 μg of bovine serum albumin per ml/20 mM Tris·HCl, pH 7.5, and tested at a final dilution of 1:200. (*B*) $NaDodSO_4$/PAGE analysis of selected fractions from the C_4 chromatography shown in *A*. Half of each fraction was dried, redissolved in $NaDodSO_4$/2-mercaptoethanol, heat-denatured, and electrophoresed in a 14% polyacrylamide gel which was subsequently silver-stained. The position of each molecular mass marker (in kDa) is indicated by an arrow. (*C*) DNA synthesis in BALB/MK cells triggered by the fractions analyzed in *B*. Activity is expressed as the fold stimulation over background, which was 100 cpm.

judged by silver-stained $NaDodSO_4$/PAGE (data not shown) but provided a far better recovery of activity (Table 1). The TSK-purified material was used routinely for biological studies as it had a higher specific activity. In both instances, the profile of mitogenic activity was associated with a distinct band on $NaDodSO_4$/PAGE that appeared to be indistinguishable in the two preparations.

Physical and Biological Characterization of the Growth Factor. The purified factor had an estimated molecular mass of 28 kDa based on $NaDodSO_4$/PAGE under reducing (Fig. 2) and nonreducing conditions (data not shown). This value was in good agreement with its elution position on two different sizing columns run in solvents expected to maintain native conformation [TSK G3000SW (Fig. 3) and Superose-12 (data not shown)]. From these data, the mitogen appears to consist of a single polypeptide chain with a molecular mass of 25–30 kDa.

Its heat and acid lability were demonstrated by using the BALB/MK mitogenesis bioassay. While activity was unaffected by a 10-min incubation at 50°C, it was reduced by 68% after 10 min at 60°C and was undetectable after 3 min at 100°C. Exposure to 0.5 M acetic acid for 60 min at room temperature resulted in a decline in activity to 14% of the control. In comparison, the mitogenic activity of EGF was not diminished by any of these treatments.

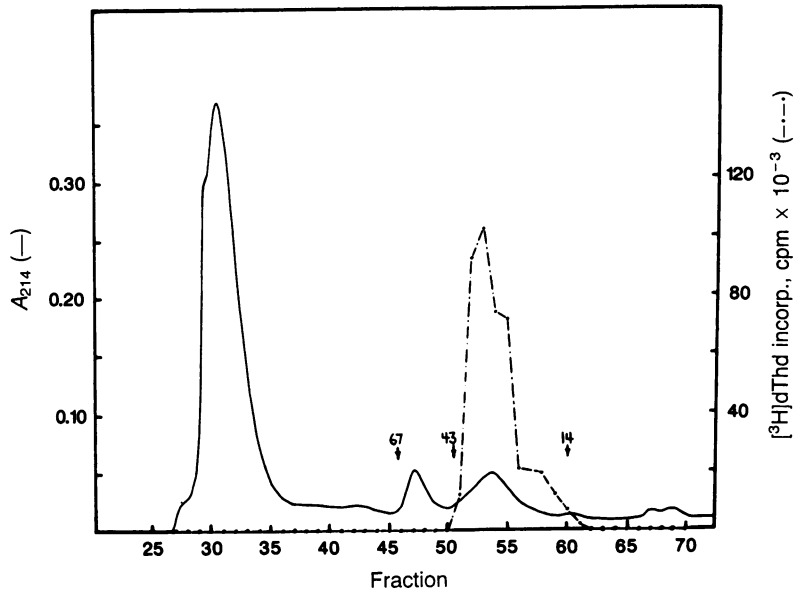

FIG. 3. TSK G3000SW chromatography of the BALB/MK mitogenic activity. Approximately 50 μl of a Centricon microconcentrator-processed 0.6 M NaCl pool from HSAC was loaded onto a GlasPac TSK G3000SW column (8 × 300 mm), previously equilibrated in 20 mM Tris·HCl, pH 6.8/0.5 M NaCl, and was eluted as 0.2-ml fractions at a flow rate of 0.4 ml/min. Aliquots of 2 μl were transferred to microtiter wells and diluted to a final volume of 0.2 ml for assay of [^{3}H]thymidine incorporation (incorp.) in BALB/MK cells. The elution positions of molecular mass markers (in kDa) were as indicated by the arrows.

Table 1. Growth-factor purification

Purification step	Protein, mg	Total activity,* units	Specific activity,* units/mg
Conditioned medium (10 liters)	1.4×10^{3}†	2.5×10^{4}	1.8×10^{1}
Ultrafiltration retentate	1.3×10^{3}†	3.2×10^{4}	2.5×10^{1}
HSAC 0.6 M NaCl pool	0.73‡	1.6×10^{4}	2.2×10^{4}
TSK G3000SW	8.4×10^{-3}‡	2.7×10^{3}	3.2×10^{5}
C_4 HPLC	6.1×10^{-3}‡	2.1×10^{2}	3.4×10^{4}

Recoveries were calculated by assuming that all of the mitogenic activity in the starting material was due to the isolated factor.
*One unit of activity is defined as half of the maximal stimulation of thymidine incorporation induced by TSK-purified growth factor in the BALB/MK bioassay. Approximately 3 ng of the TSK-purified factor stimulated 1 unit of activity in this bioassay.
†Protein was estimated by using the Bradford reagent from Bio-Rad (23).
‡Protein was estimated by using $A_{214}^{1\%} = 140$.

The dose–response curve for the purified growth factor depicted in Fig. 4 illustrates that as little as 0.1 nM led to a detectable stimulation of DNA synthesis. Thus, the activity range was comparable to that of the other growth factors analyzed to date. A linear relationship was observed in the concentration range 0.1–1.0 nM, with maximal stimulation of 600-fold observed at 1.0 nM. The novel factor consistently induced a higher level of maximal thymidine incorporation than did EGF, aFGF, or bFGF (Fig. 4).

Its distinctive target-cell specificity was demonstrated by comparing it on a variety of cell types with other growth factors known to possess epithelial cell mitogenic activity. The newly isolated factor exhibited a strong mitogenic effect on BALB/MK cells and induced demonstrable thymidine incorporation in the other epithelial cells tested (Table 2). In striking contrast, the factor had no detectable effects on mouse (or human, data not shown) fibroblasts or human

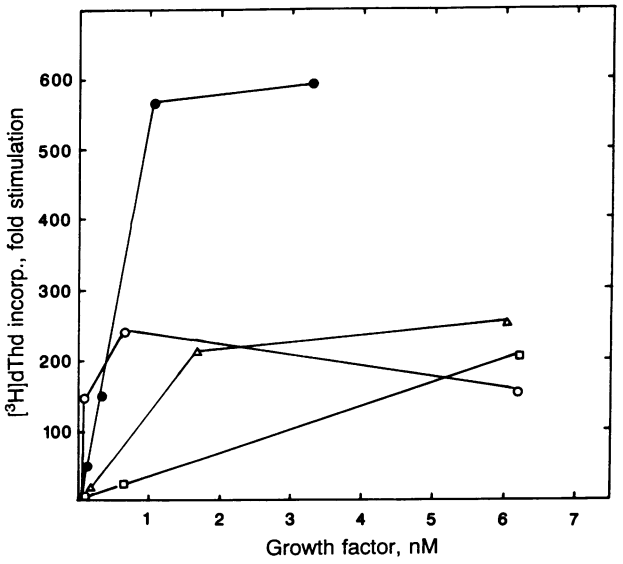

FIG. 4. Comparison of BALB/MK DNA synthesis in response to TSK-purified mitogen and other growth factors. Incorporation (incorp.) of [^{3}H]thymidine into trichloroacetic acid-insoluble DNA, expressed as fold stimulation over background, was measured as a function of the concentration of the indicated growth factors. The background value with no sample added was 150 cpm. The results represent mean values of two independent experiments. Replicates in each experiment were within 10% of mean values. ●, TSK-purified mitogen; △, EGF; □, aFGF; ○, bFGF.

Table 2. Target-cell specificity of growth factors

Growth factor	Fold stimulation of thymidine incorporation: Epithelial cell line BALB/MK	B5/589*	CCL208	Fibroblast NIH 3T3S	Endothelial cell line†
KGF	500–1000	2–3	5–10	<1	<1
EGF	100–200	20–40	10–30	10–20	ND
TGF-α	150–300	ND	ND	10–20	ND
aFGF‡	300–500	2–3	5–10	50–70	5
bFGF	100–200	2–3	2–5	50–70	5

Comparison of maximal thymidine incorporation stimulated by KGF and other growth factors in a variety of cell lines, expressed as fold stimulation over background. These data represent a summary of four different experiments. ND, not determined.
*The mammary cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum and 4 ng of EGF per ml. When maintained in serum-free conditions, the basal medium was DMEM.
†Human saphenous vein cells.
‡Maximal stimulation by aFGF required the presence of heparin (Sigma) at 20 μg/ml.

saphenous vein endothelial cells. By comparison, TGF-α and EGF showed good activity on fibroblasts, while aFGF and bFGF were mitogenic for endothelial cells as well (Table 2). Because of its specificity for epithelial cells and the sensitivity of keratinocytes in particular, the mitogen was provisionally designated "keratinocyte growth factor" (KGF).

To establish that KGF not only would stimulate DNA synthesis but also would support sustained cell growth, we attempted to grow BALB/MK cells in a fully defined, serum-free medium supplemented with this growth factor. KGF served as an excellent substitute for EGF but not for insulin (or insulin-like growth factor I) in this chemically defined medium (Fig. 5). Thus, KGF acts through the major signaling pathway shared by EGF, aFGF, and bFGF for proliferation of BALB/MK cells (14).

Microsequencing Reveals a Unique N-terminal Amino Acid Sequence of KGF. To further characterize the growth factor, ≈150 pmol of C_4-purified material was subjected to sequence analysis. A single sequence was detected with unambiguous assignments made for cycles 2–13 as follows: Xaa-

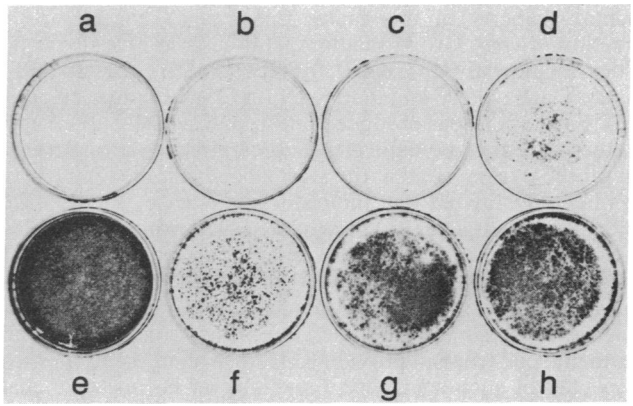

FIG. 5. Comparative growth of BALB/MK cells in a chemically defined medium in response to different combinations of growth factors. Cultures were plated at a density of 2.5×10^4 cells per dish on poly(D-lysine)/fibronectin-precoated 35-mm Petri dishes in 1:1 (vol/vol) Eagle's minimal essential medium/Ham's F-12 medium supplemented with transferrin, Na_2SeO_3, ethanolamine, and the growth factors indicated below. After 10 days, the plates were fixed and stained with Giemsa. (*a*) No growth factor. (*b*) EGF alone. (*c*) Insulin alone. (*d*) KGF alone. (*e*) EGF and dialyzed fetal calf serum (final concentration, 10%). (*f*) KGF and EGF. (*g*) KGF and insulin. (*h*) EGF and insulin. Final concentrations of the growth factors were as follows: EGF, 20 ng/ml; insulin, 10 μg/ml; and KGF, 40 ng/ml.

Asn-Asp-Met-Thr-Pro-Glu-Gln-Met-Ala-Thr-Asn-Val. High background noise precluded an assignment for the first position. The initial yield was estimated to be 70 pmol, with a repetitive yield of 90%. A computer search using the FASTP program (24) revealed that the N-terminal amino acid sequence of KGF showed no significant homology to any protein in the National Biomedical Research Foundation data bank (May 1988, Release 16.0).

DISCUSSION

In the present study, we identified a human growth factor that has a distinctive specificity for epithelial cells. By using ultrafiltration, HSAC, and RP-HPLC or TSK molecular sieve chromatography, we isolated a quantity sufficient to permit detailed characterization of the physical and biological properties of this molecule. A single silver-stained band corresponding to a molecular mass of 28 kDa was detected in the active fractions from RP-HPLC, and the intensity of the band was proportional to the level of mitogenic activity in these fractions. A band indistinguishable from that obtained by RP-HPLC was seen in the active fractions from TSK chromatography. The purified protein stimulated DNA synthesis in epithelial cells at subnanomolar concentrations but failed to induce any thymidine incorporation in fibroblasts or endothelial cells at comparable or higher concentrations (up to 5 nM). Its unique target-cell specificity and N-terminal amino acid sequence lead us to conclude that it represents a previously unreported growth factor.

In a chemically defined medium, the purified factor was able to complement the insulin-like growth factor I/insulin growth requirement of BALB/MK cells and, therefore, must act through a signal-transduction pathway shared with EGF, TGF-α, and the FGFs. Moreover, the purified factor was more potent than any of the known epithelial cell mitogens in stimulating thymidine incorporation in BALB/MK cells. Preliminary evidence indicates that this factor is also capable of supporting proliferation of secondary human keratinocytes (our unpublished observations). In view of its preferential activity on the keratinocytes compared with other epithelial cells, we have provisionally named it KGF.

The ability of KGF to bind heparin may signify a fundamental property of this factor that has a bearing on its function *in vivo*. Growth factors with heparin-binding properties include aFGF (20–22), bFGF (19, 22), granulocyte–macrophage colony-stimulating factor (25), and interleukin 3 (25). Each of these is produced by stromal cells (25–27). Such factors appear to be deposited in the extracellular matrix or on proteoglycans coating the stromal cell surface (25, 28). It has been postulated that their storage, release, and contact with specific target cells are regulated by this interaction (25, 28). While mesenchymal-derived effectors of epithelial cell proliferation have also been described (29–31), their identities have not been elucidated. Its heparin-binding properties, release by human embryonic fibroblast stromal cells, and epithelial cell tropism provide KGF with all of the properties expected of such a paracrine mediator of normal epithelial cell growth. The partial amino acid sequence determined for this new growth factor should aid in efforts to molecularly clone its coding sequence and ascertain its relationship, if any, to known families of growth factors as well as its possible role in diseases characterized by epithelial cell dysplasia or neoplasia.

Note. Using oligonucleotide probes based on the N-terminal sequence reported in this manuscript, we have isolated clones encoding KGF from an M426 cDNA library. Sequence analysis reveals that KGF is a distinctive molecule with significant structural homology to the other five known members of the FGF family (32–35) (unpublished data).

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